An Appropriate Constitutive Promoter for Over Expression in *Dendrobium* Protocorms and Flowers

Chidchanok Suwannaketchanatit[1], Pornsuk Chaisuk[1], Chitrapan Piluek[2], Surin Piyachokanakul[1] and Pattana Srifah Huehne[1]*

ABSTRACT

Three different constitutive promoters, rice Act-1, CaMV35S, and maize Ubi-1, were determined the efficiency to control spatial trans-gene expression in protocorms and sepal tissues of *Dendrobium* orchids. Each of the promoters was fused to *B-glucuronidase* (*gus*) gene and Nos terminator in the plant expression vectors including pActin1-D, pAHC27, and pP2K7, respectively. The plasmid vectors were individually bombarded into active dividing protocorms and fresh sepals of *Dendrobium* Jacquelyn Thomas orchid. Transient *gus* gene expression assayed after 3 days of bombardment. The results demonstrated that all tested promoters were able to drive *gus* gene expression in the orchid protocorms with different level. Ubi-1 promoter conferred the most efficiency promoter in protocorms at the highest level of *gus* gene expression. Whereas, GUS activity was distinctly observed under the control of Act-1 and CaMV35S in the *Dendrobium* sepal tissues. Both Act-1 and CaMV35S promoter performed well in the vascular tissue of flower. Surprisingly, the Ubi-1 promoter had no function in sepal of *Dendrobium* orchid flowers.

Key words: *Dendrobium* orchid, promoter, Actin, bombardment, transformation

1 Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
Tel.: +66-2-9428716, Fax: +66-2-5795528

2 Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
Tel.: +66-2-5790308, Fax: +66-2-5795528

* Corresponding author:
Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
Tel.: +66-2-9428716 ext 121, Fax: +66-2-5795528
e-mail: fscipns@ku.ac.th

INTRODUCTION

To date, genetic engineering has been commonly used for introduction of attractive genes into plant for improvement including orchids. Because of the desirability of global marketing, many traits were included in biotechnology programs such as novel color and shape, long vase life, and pests and disease resistance. Since the success of genetic plant transformation is not only gene integration into chromosome of transgenic plant but the gene also is a high level of expression in the target tissue, the suitable promoter is an important factor to drive spatial and temporal gene expression. Several constitutive promoters were available for control trans-gene expression in plant such as CaMV35S promoter from the 5′ region of *cauliflower mosaic virus* gene (Guilley *et al*., 1982), Act1-D promoter from rice *actin* gene (McEloy *et al*., 1990), Adh-1 promoter from *alcohol dehydrogenase* gene (Ellis *et al*., 1987), and Ubi-1 promoter from *ubiquitin* gene (Toki *et al*., 1992). For orchid transformations, the constitutive promoters reported for driving gene expression were Act-1, CaMV35S, and Ubi-1. In particular, CaMV35S is widely used to control trans-gene expression in several transgenic orchid cultivars. Nevertheless, previous reports revealed that CaMV35S promoter was not active in all cell types of tobacco (Benfey and Chua, 1989), rice (Terada and Shimamoto, 1990; Wilkinson *et al*., 1997) and also low-level expression in transgenic monocots (Zhang *et al*., 1991; Christensen, 1992; Chowdhury, 1997). On the contrary, the promoter isolated from monocotyledon showed higher activity in transgenic monocotyledon such as in rice (Toki *et al*., 1992; Cornejo *et al*., 1993) and in maize (Zhong, 1996; Christensen *et al*., 1992). As noted earlier, orchid transformation showed high potential of CaMV35S promoter for driving expression of the selectable marker gene in protocorm during selection (Anzai *et al*., 1996, Yu *et al*., 1999; Tee *et al*., 2003). However, many attractive traits for orchid improvement have been focused on the flower such as to create a novel color and shape; there was no any information on the activity of the potential promoter controlling the gene expression in flower. Therefore, this paper reported on the efficient promoters for ubiquitous transient gene expression in protocorm and flower of *Dendrobium* orchid through particle bombardment technique.

MATERIALS AND METHODS

Tissue culture medium test

Young shoots 3-4cm in length of *Dendrobium* Jaquelyn Thomas orchids were soaked in 70% ethanol for 10min and 2.5% sodium hypochlorite for 20min followed by three thorough washing in sterilized distilled water. Shoot tips and auxiliary buds (2mm) were excited from the shoots, after removal of the leaf sheaths. The tissues were cultured in a modified VW medium (Vacin and Went, 1949) with shaking at 100rpm under artificial light of 35$\mu molm^{-2}s^{-1}$ with a light/dark cycle of 16/8h at 25^{o}C. Protocorms were induced and sub-cultured to fresh medium about 14d intervals. After 90d of subculture, the second protocorms were used for the growth and regeneration tests in VW, 1/2MS (Murashige and Skooge, 1962*)*, MS+VW, and KC (Knudson, 1946) medium supplemented with 20g/l sucrose, 2g/l active charcoal, and 8g/l agar.

Plant material preparation

The protocorms of *Dendrobium* orchid obtained from young apical bud were sub-cultured to produce new active dividing tissue in liquid VW for 21d (Figure1). The protocorms were pretreated for decreasing cell osmotic pressure by culturing on MS medium supplemented with 0.8% manital, 0.2% sucrose, and 0.8% agar for 4h before particle bombardment. Fresh sepals of *Dendrobium* Jaquelyn Thomas flower collected from Rapee Sagarik Orchid Garden, Kasetsart University, Thailand, were used for evidence expression. They were surface-sterilized by immersing 15min in 10% of Clorox and rinsed three times with sterile distilled water. The sterilized sepals were placed on wetted 3MM paper in Petri-dish.

Plasmids

Three plant vectors, pAct1-D (kindly provided by Prof. Ray Wu), pAHC27 (gift of Prof. Hirofumi Uchimiya), and p2K7 (gift of Dr. Jim Dale), contain rice Act-1 promoter (McElroy *et al*., 1990), maize Ubi-1 promoter (Toki *et al*., 1992) and CaMV35S promoter (Guilly *et al*., 1982), respectively (Figure2). Each of the promoters fused with β-glucuronidase (*gus*) gene and Nos terminator was compared their ability in transient *gus* gene expression in *Dendrobium* protocorm and flower.

Microprojectile bombardment

Determining the promoter for gene expression in *Dendrobium* protocorms, the gold microparticles coated with each of the plasmid DNAs were prepared as described by Liang (1998). The coated microparticles were accelerated into protocorms of *Dendrobium* with the He gas pressure of 4,100kpa and the target distance of 10cm with different vacuum chamber (-20, -25, or -40kpa) by particle inflow gun. The bombarded tissues then were cultured under a 16h photoperiod by cool white light at 25^{O}C. To determine the promoter for gene expression in orchid flower, coated gold microparticles were bombarded to the orchid sepals of *Dendrobium* Jaquelyn Thomas as following parameters: the He gas pressure of 4,100kpa, the target distance of 12cm, and vacuum chamber at 25inch Hg using PHD 1000/Helium biolistic device (Bio-Rad Laboratories, Hercules, California).

Gus histochemical assay

After 3d of bombardment, both treated protocorms and sepals of flowers were examined for GUS activity by using histochemical staining (Jefferson *et al*., 1987). The tissues were immersed in 5-bromo-4-chloro-3-indonyl-B-D-glucuronic acid (X-gluc) buffer containing 1mM X-gluc, 100mM sodium phosphate buffer, pH 8.0, 0.5mM potassium ferricyanide, and 0.5mM potassium ferrocyanide and incubated overnight at 37^{o}C. After staining, the chlorophyll of bombarded tissues then were removed using 70% ethanol in order to examine and count the blue spots occurred on the protocorms and the sepals.

RESULTS

Plant culture

The second *Dendrobium* protocorms well responded to those four kinds of solid medium including VW, 1/2MS, MS+VW and KC. Within the first 14d for sub-cultured protocorms, the development of protocorms in each medium appeared similar with the same rate of growth. After 21d, the protocorms in 1/2MS, MS+VW began to regenerate into multiple small shoots unlike in VW and KC. They continuously multiplied as protocorms forming in a big lump shape. However, the protocorm type in KC medium looked rather different from the other with dark green color, succulent, clear and shiny lump. The stages of *Dendrobium* protocorm development in each medium have shown in Table 1. The 1/2MS medium seems to be a suitable medium for orchid regeneration. It can produce the plantlets

within 60d of tissue transferring. The active protocorm sub-culturing in VW were selected and subjected to the next step of transient gene expression test.

Table1 Growth and development of *Dendrobium* protocorms like bodies (Plb) on three kinds of medium.

Week*	**Size and developments of protocorms**			
	VW	**1/2MS**	**MS+VW**	**KC**
1	0.2-0.5cm Plb	0.2-0.5cm Plb	0.2-0.5cm Plb	0.2-0.5cm Plb
2	0.5-2 cm Plbs	0.5-2 cm Plbs	0.5-2cm Plb	0.5-2cm Plb
3	1.5-3 cm Plbs	tiny shoots	lots of tiny shoot	2 cm shiny Plb
4	tiny shoots	4-6 leaf	3.5cm Plb+shoot	smooth shiny Plb
6	Plb+4-6 leaf	small plantlet	Plb+shoot+leaf	shinyPlb+2-4 leaf
8	Plb+plantlet	rooting plantlet	plantlet	shinyPlb+2-4 leaf

* the number of weeks after transferring to tested media

Effecting promoters to protocorm expression

To compare the efficiency of each constitutive promoters, Act-1 (pAct1-D), CaMV35S (p2K7) and Ubi-1 (pAHC27), controlling *gus* gene expression in orchid protocorms, the bombarded protocorms were determined transient GUS activity using histochemical assay. The results showed that all of the promoters tested for their functions were able to drive *gus* trans-gene in the *Dendrobium* orchid protocorms under the optimal condition. At the level of vacuum chamber of -25kPa, the highest transient *gus* expression was detected in the protocorm under the control of Ubi-1 promoter (Table2). While *gus* gene expression driven by Act-1 and CaMV35S promoters were 41-60 % and 61-80 %, respectively, in average of the total area cover on the protocorm surface. Figure3B revealed that *gus* expression in transformed protocorms driven by Ubi-1 promoter were entirely occurred. The efficiency of Act-1 promoter conferred lower expression (Figure3C) than CaMV35S promoter (Figure3D). At the others different levels of vacuum chamber, however, Act-1 promoter still able to control the expression of *gus* gene but CaMV35S promoter is less. Moreover, no any of the *gus* expression in the protocorms driven by CaMV35S promoter with the level of vacuum in chamber at -40kPa was obtained (Table2). These results demonstrated that Ubi-1 and Act-1 promoter contained efficient region for expression in orchid protocorm.

Table 2 Effecting constitutive promoters to control *gus* gene expression in bombarded protocorms of *Dendrobium* Jaquelyne Thomas at different level of vacuum chamber.

Plasmid	Promoter	Vacuum Chamber (kPa)	GUS activity [a]
pAHC27	Ubi-1	-20	+++
		-25	++++
		-40	++
pAct1-D	Act-1	-20	+++
		-25	++
		-40	+
p2K7	CaMV35S	-20	++
		-25	+++
		-40	-

a: Determining transient GUS activity by area and intense of blue spots in cells of *Dendrobium* protocorms; ++++: 81-100%, +++: 61-80%, ++: 41-60%, +: at less 40%, and -: no detected blue spots.

Effecting the promoters to floral expression

Each of the three plasmids consisting of a constitutive promoter, Act-1, Ubi-1, and CaMV35S, was also delivered into fresh sepal of *Dendrobium* Jaquelyne Thomas to evaluate for floral expression. Bombarded sepal tissues were determined for *gus* gene transient expression by histochemical assay one day after bombardment. The results revealed that the ability of each promoter rather differed in driving *gus* trans-gene between protocorm and floral orchid tissues. The Act-1 and CaMV35S promoters were active in the *Dendrobium* sepal, while the Ubi-1 and Act-1 promoter were highly active in the *Dendrobium* protocorm. However, *gus* gene expression under the control of Act-1 promoter (Figure4A) in the examined sepal was more activity than under the control of CaMV35S (Figure4B). The high level of expression with Act-1 promoter appeared throughout the sepal base. Similarly, CaMV35S promoter worked particularly well within the vascular tissue (Figure4C). Interestingly, no any blue spots were observed in orchid sepal under the control of Ubi-1 promoter (Figure4D). The results demonstrated that the maize Ubi-1 promoter might not be active in the sepal and the vascular cells of orchid flower.

DISCUSSION

In order to determine a potential promoter to specifically control gene expression, two types of *Dendrobium* orchid tissue, protocorm and sepal, were used to evaluate the efficiency of three kinds of a constitutive promoter. Rice Act-1, CaMV35S and maize Ubi-1 promoters were constructed in pAct1-D, pAHC27 and p2K7 plant vectors, respectively, for *gus* gene transient expression assay in those orchid tissues. In this study showed that rice Act-1 and maize Ubi-1 promoters controlled the high level of GUS expression in orchid protocorms comparing with CaMV35S promoter. Although, previous reports revealed that CaMV35S promoter had been an efficient promoter for control expression of *gus* gene in callus of *Phalenopsis* (Anzai *et al*., 1996) and of *Dendrobium* (Tee *et al*.,2003), and in protocorms of *Dendrobium* hybrid (Yu *et al*., 1999).

However, previous reports demonstrated that the Ubi-1 promoter is higher potential than CaMV35S promoter in monocotyledon plants, including maize and barley (Schledzewshi and Medel, 1994), and oil palm (Chowdhury *et al*., 1997). The GUS expression driven by Ubi-1 promoter was more 10 fold-level of activity than expression driven by CaMV35S promoter in maize protoplast (Christensen, 1992). Moreover, the highest GUS expression under the control of Act-1 and Ubi-1 promoters was obtained in rice transformation (Zhang, 1991**;** Zhongyi *et al*., 1997). Similarly, in our studies, the *gus* expression in *Dendrobium* orchid protocorms driven by CaMV35S promoters was lower level compared to Ubi-1 and Act-1 promoters, replying that the transcription factor presenting in orchid protocorms might be more sufficient to recombine with *cis*-element of the promoter isolated from monocots, Ubi-1 and Act-1, than the promoter isolated from dicots, CaMV35S.

On the contrary, in the sepals of *Dendrobium* orchid, Act-1 and CaMV35S promoters were able to drive *gus* gene with high level of expression though their patterns of expression in the sepal base and the vascular tissues of orchid flowers was similar. In transgenic maize, the Act-1 promoter gave the high level expression in shoot and floral meristematic tissues (Zhong *et al*., 1996) and in the most cell types of rice (Zhang *et al*., 1991); whereas, CaMV35S promoter is known as an efficient promoter in dicotyledon. All cells of transgenic rice was not detected its expression (Tereda and Shimamoto, 1990; Cornejo, 1993). Wilkinson *et al*. (1997) demonstrated that CaMV35S promoter could active in leaves but not in pollen of all *Arabidopsis* lines. In anther and pollen tissues, CaMV25S expression was strong in transgenic tobacco but limited in transgenic petunia. Moreover, CaMV35S

promoter was expressible in most cells and tissue types of transgenic cotton (Sunilkumar *et al*., 2002) and strawberry (Toki *et al*., 1992), implying that the level of CaMV35S expression might depend on the tissue types.

As the result shown the high efficient of Ubi-1 promoter in protocorms but not in the flower, demonstrating that recognize of *cis*-elements of Ubi-1 promoter and *trans*-acting factor presenting in the flower might be limited. Ubi-1 promoter was highly potential to express in active dividing and growing cell (Carnejo *et al*., 1993; Stephen, 2004). A potato ubiquitin promoter, Ubi-3, showed the highest expression in meristematic tissue and declined during leaf expansion in transgenic potato and rose (Gurbarino and Belknap, 1994). In transgenic rice, Ubi-1 promoter showed highest expression in the root tips and lowest in the mature root. It is implied that Ubi-1 promoter plays a key role in cell division of eukaryote (Takimoto, 1994; Plesse, 2001). Similarly, in these results showed the highest expression in the protocorm containing actively dividing cells but the lowest expression was observed in mature flower containing differentiated mature cells.

CONCLUSION

The choice of promoters used for driving trans-gene expression is one of the most critical successes in plant genetic transformation. The results demonstrated that the promoters affected to gene expression in *Dendrobium* Jaquelyn Thomas tissues. Rice Act-1 promoter contains highly sufficient regions to recombine with transcription factor presenting in the saprophyte and gametophyte tissues for driving gene expression. Although, the Act-1 promoter does not give the highest activity as Ubi-1 promoter in *Dendrobium* protocorm, it is high potential in driving gene expression in tested tissues, protocorms and sepal of mature flower. Whereas, maize Ubi-1 promoter showed the highest level of expression in protocoms, but its expression was not detected in the flower. In addition, CaMV35S promoter was constitutive promoter to drive gene expression in protocorm as well, but CaMV35S promoter conferred lower expression than Act-1 promoter in the sepal of flower. Consequently, rice Act-1 would be highly potential promoter for orchid flower improvement through protocorm in the future.

ACKHOWLEDGEMENTS

We are grateful to Prof. Dr. Ray Wu (Cornell University, Ithaca, NY) for the gift of the pActin1-D plasmid, Prof. Hirrofumi Uchimiya (University of Tokyo, Tokyo, Japan) for the gift of the pAHC27, and Dr. Jim Dale (Queensland University of Technologgy, Austraria) for providing p2K7 plasmids. This work was supported by grants from the Thailand Research Fund and National Center for Genetic Engineering and Biotechnology, Thailand.

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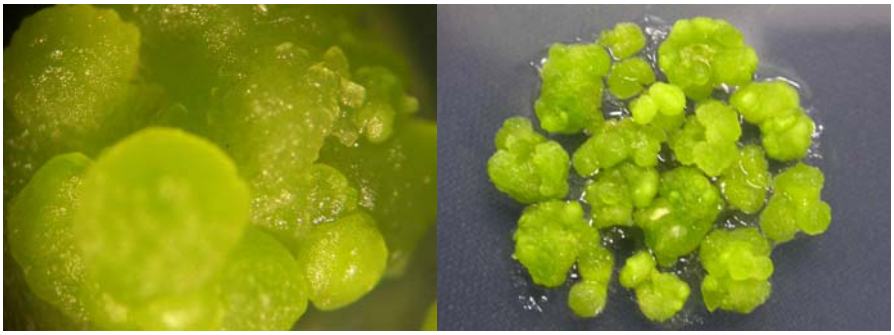

Figure 1 Type of the actively dividing cells of *Dendrobium* protocorms using for gene transformation.

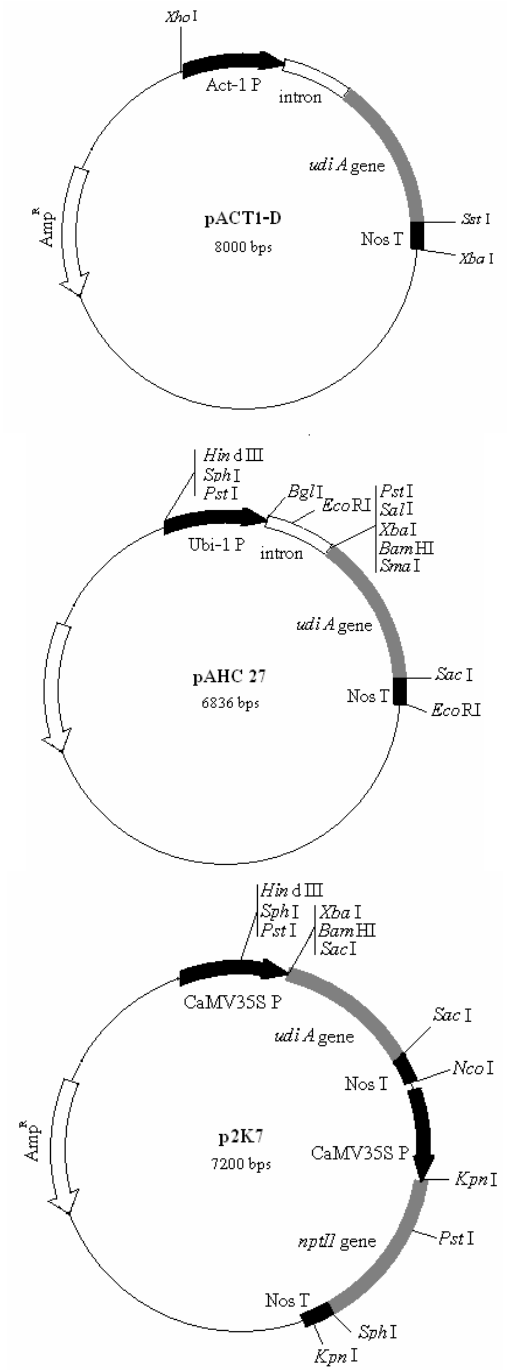

Figure 2 Schematic the plasmids used to determine transient GUS activity.

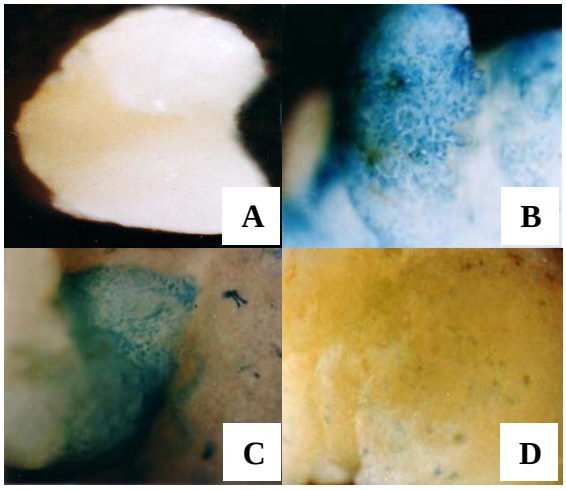

Figure 3 Transient GUS activity in protocorm like bodies of *Dendrobium* orchid with different promoters at the level of vacuum chamber of -25kPa; un-bombarded plb (A);Ubi-1 promoter (B); Act1-D promoter (C); CaMV35S promoter (D).

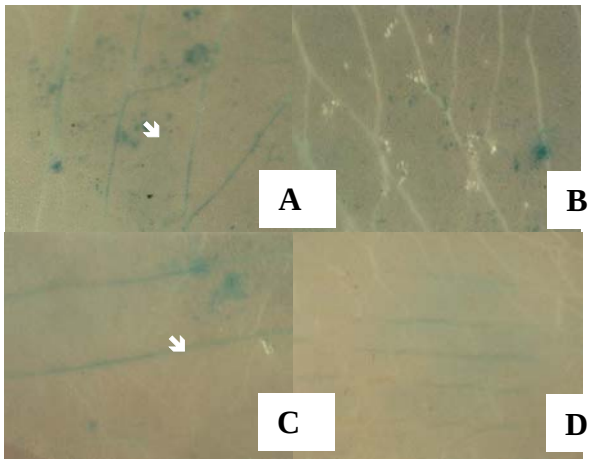

Figure 4 Transient GUS activity in sepal *Dendrobium* orchid with different of the promoter: Act1-D promoter (A);CaMV35S promoter (B,C); Ubi-1 promoter (D). White arrow indicated blue color of GUS activity in vascular tissue.